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Study of liver toxicity and DNA damage due to exposure to the pesticide Mancozeb in an experimental animal model – A pilot model

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Abstract. – OBJECTIVE: Mancozeb is one of the most widely used Ethylenebisdithiocarbamates fungicides in Brazil. A pilot experimental model was created to evaluate its potential genotoxic effect.

MATERIALS AND METHODS: An experimental study was performed with 27 male Wistar rats (3 groups). The Control Group received a saline solution, while Intervention Groups I and II received 250 mg/kg and 500 mg/kg of Mancozeb, respectively, once a week for 12 weeks. Anthropometric measurements were carried out and the marker of biological exposure was dosed. Biochemical tests, evaluation micronucleus count, comet and oxidative stress markers assay, and histological assessment of the liver were also performed.

RESULTS: The hepatotoxic effect of Mancozeb was confirmed by anthropometric measurements, genotoxicity, and oxidative stress. Statistically significant results were found when the exposed groups were compared to the control group.

CONCLUSIONS: The results were supported by histomorphometric infiltration and ballooning in the treated groups. The experimental model effectively demonstrated the deleterious effects of Mancozeb on the liver.

Ethylenebisdithiocarbamates, Mancozeb, Oxidative stress, Genotoxicity, Liver.

Introduction

Brazilian agriculture has developed to such an extent in the last 40 years that the country is set to become one of the world's largest food suppliers. This sector has played a major role in the Brazilian economy due to high grain production throughout all macro-regions of the country. However, in order to maintain this production, the agricultural sector carries out extensive use of chemical inputs, such as fertilizers and agricultural pesticides, making Brazil one of the main pesticide consumers in the world. Due to the expansive use and adverse events known from the literature[2], pesticide adoption has had a strong social impact and is considered a challenge to world public health.

Pesticides were developed to avoid pest invasion of crops and to protect the consumer, as a public health[3] aspect. Among the most widely used classes of pesticides there are fungicides, which are appropriate to prevent or eradicate fungal infections in plants or seeds.

Ethylenebisdithiocarbamates (EBDCs) are a group of fungicides that have been widely utilized around the world since the 1940s[4,5]. Among the EBDCs there are Mancozeb, Maneb, Zineb and Methyran[6]. Manganese Ethylenebis (Mancozeb), according to the literature, is classified as having low toxicity, but has proved[7] to have caused adverse effects in humans. Its toxicity was induced by the activation of free radicals and suppression of antioxidants[8].

Innes et al[9] (1969) demonstrated that chronic exposure to Mancozeb (18 months) increases the incidence of adenoma and hepatocellular carcino-

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ma in female and male rats. Ahmed et al[10] (2017) showed evidence of different alterations in the biochemical and hematological parameters. Other authors, such as Yahia et al[11] (2015), found similar results, mainly involving the transaminases.

Fungicide toxicity is often related to the formation and increase of reactive oxygen species (EROS), resulting in oxidative damaging products and/or changes in the levels of antioxidants and enzymatic systems for eliminating EROS[12], creating an imbalance called oxidative stress[13]. Exposure to pesticides has been associated with the induction of oxidative stress in multiple systems[14]. Some authors, such as Atamaniuk et al[15] (2014), focused their research on the evaluation of oxidative stress, which was demonstrated by different enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)[15]. Besides the effects of oxidative stress following exposure to Mancozeb, the evaluation of genotoxicity from pesticides triggers chronic effects harmful to humans. These effects begin with cellular damage and potentially cause the development of teratogenesis and cancer[16,17]. Among the methods to detect damage to DNA we can cite the micronucleus (MN) test[ ] icity caused by Mancozeb has been repor[ ] in various experimental studies[19-21], which sho[ ] evidence of the suspicion of carcinogenicity rats[19] and induction of damage t[ ] expose cells *in vitro* through oxidat[ ] mec[ ]sms[20,21]. Clinical studies aiming to [ ]luate t[ ]enotoxicity of EBDCs are scarc[ ] the li[ ] the histological eval[ ]n [ ]er is rarely described. The wo[ ]y Pirozz[ ]l[22] (2016) is one of the few r[ ]e studies, in[ ]h the degrees of liver [ ]to[ ]sociated wi[ ]exposure to Mancoz[ ] are evalu[ ] concluding that the fungicid[ ]reased the i[ ]r of intracellular lipid d[ ]ets.

D[ ]to the [ ]ible damage caused by Manganese [ ]th[ ]ebis (M[ ]ncozeb) following prolonged [ ]ure th[ ]n contact with the con[ ]ant [ ]ls[ ] food residues[23], we chose to [ ]ly ther[ ]otoxic effects in detail in an ex[ ]mental m[ ]del.

Materials and Methods

An experimental study was performed with 27 male Wistar rats weighing 280 to 300 g. The animals were placed in boxes with 3 rats in each box, on a wood shavings bed, and fed a standard diet and water *ad libitum*. The rats were maintained on a 12-hour light/dark cycle, at a temperature of 22 ± 1ºC. The maximum dose defined for this model was 500 mg/kg, based on the lethal dose of Mancozeb. This dose corresponds to [ ] the lethal dose[1].

Experimental Design

The animals were divided r[ ]ndom[ ] three groups:

- Control Group (CG): [ ]ats that rece[ ] saline solution (0.9[ ]NaCl) [ ]r the sa[ ] frequency as the ot[ ]our[ ]ring th[ ] same period.
- Intervention [ ]up I (M[ ]9 r[ ] that received a [ ]f Mancoze[ ]ane® NT) (250 m[ ]g/d[ ]issolved i[ ] saline solution (0.9% NaCl) [ ]a final volume of 2 ml/K[ ]nistered by [ ]ge, once a week, for [ ]eeks.
- [ ]ervention Gr[ ]up II (MZ2): 9 rats that re[ ]ived a dos[ ]f Mancozeb (Dithane® NT, [ ]y AgroSc[ ]es Industrial Ltda, Jacareí/[ ]ulo, B[ ]l) (500 mg/Kg/day)[10] dissolved in a [ ]ution (0.9% NaCl), with a volume of 2 ml/Kg administered by gavage, once a [ ]k, for 12 weeks.

This model proposed to mimic the exposure to Mancozeb to winegrowers in the state of Rio Grande do Sul, Brazil. This is characterized by farmers who apply this fungicide annually, from October to December, with a total of approximately 12 weeks of exposure. This work is a pilot study of a clinical model.

Anthropometric Measurements and Procedures

Anthropometric measurements, such as weight, abdominal circumference, and naso-anal length, were taken weekly and at the end of the experiment.

Approximately (~2 mL) of urine were collected two days before euthanasia, through metabolic cages, to evaluate the biological indicator of exposure: Ethylenethiourea (ETU).

During the experiment, in week 10, one of the rats in group MZ1 died because of alimentary bronchoaspiration, without any histological change in the liver. After the experiment ended, the animals were anesthetized with isoflurane (Instituto Biochimico Ind. Farm. Ltda. Penedo/Cordovil, Rio de Janeiro, Brazil) at a concentration of 5% diluted in oxygen 100%. After confir-

mation of the anesthetic level, the animals were exsanguinated by the transcardiac route to collect blood and organs, and some of them were stored under appropriate conditions.

Biochemical and Hematological Analyses

The following were analyzed: total bilirubin (TB) and fractions – Direct (DB) and Indirect (IB) – creatinine (colorimetric method), AST and ALT (enzymatic method), and alkaline phosphatase (colorimetric kinetic method) (p-NNP - DG KC). Evaluation of blood count and platelets was made using the light absorbance/impedance/flow cytometry and acetylcholinesterase (kinetic enzymatic) methods.

Genotoxicity

After collecting peripheral blood and bone marrow from the rat femur, genotoxicity was evaluated using the Micronucleus test, following the protocol of Miller et al[24] (1997) and Comet assay. The first was performed on blood samples that were rubbed and stained with Giemsa, and then the micronuclei present on the slides were counted by two blinded researchers. The C[illegible]t assay was evaluated in the blood and liver. In the latter, the tissue was dissected and p[illegible]d in a buffered solution pH 7.4 (PBS), mixed [illegible] agarose 0.75%, and spread on slides, with a [illegible] er application of electricity fo[illegible]utes, an[illegible] neutralized after electroph[illegible]s to[illegible] finally analyzed.

Oxidative Stress

In serum and li[illegible]issue sa[illegible] lipid perox-idation was eva[illegible]using the [illegible]d of species reactive t[illegible]ob[illegible]ric acid (T[illegible]RS), followed by th[illegible]evaluation [illegible]peroxide Dismutase (SOD), C[illegible]ase (CAT), G[illegible]hione (GSH) enzymes[illegible]d by spectrophotom[illegible]try, and proteins carb[illegible]ated b[illegible]e Bradford[25] method (1976).

Histo[illegible] Anal[illegible]

After [illegible]to[illegible]nd extraction of the liver, the[illegible]er tis[illegible]s stored in formaldehyde at 10[illegible] Formald[illegible]yde solution 10% Sigma-Al-[illegible]ouis, MO, USA) for 48 hours and [illegible]aced in paraffin blocks, stained in Hematox-[illegible]n and Eosin, to evaluate liver steatosis and [illegible]Sirius Red for fibrosis, categorized in the fol[illegible]wing patterns:

A: Absence of portal fibrous expansion, perivenular or perisinusoidal fibrosis.
B: Discrete balloonization of perivenular hepatocytes, with occasional foci of inflammatory infiltrates.
C: Discrete balloonization of perivenular hepatocytes.
D: Discrete perivenular inflammatory foci[illegible]

Statistical Analysis

Normality was evaluated [illegible]using [illegible]Shapiro-Wilk test. The quantita[illegible]variable[illegible]me-dian, minimum and ma[illegible]um were des[illegible] and compared among t[illegible]groups[illegible]g non-pa[illegible]metric tests such as [illegible]ska[illegible]allis, f[illegible]llowed by Dunn-Bonferr[illegible]pos[illegible]or the [illegible]oups) and Mann-Whi[illegible], when t[illegible]oup[illegible]re compared.

Categor[illegible]va[illegible]s were pre[illegible]ted as number and percentage. [illegible]Exact Fisher's test was used[illegible]pare categ[illegible]l variables. Associations with $p \leq 0.05$ were [illegible]nsidered statistically s[illegible]ficant. A statistical analysis was performed [illegible]g the statist[illegible] program SPSS version 20.0 ([illegible] Corp., Ar[illegible]k, NY, USA).

Results

The findings of this study on anthropometric measurements were demonstrated using the Lee index and Body Mass Index (BMI). There was a statistical significance when the control group was compared to the exposed groups MZ1 and MZ2 ($p = 0.01$).

The medians of weight at the end of the experiment in each group were 527 grams in the Control Group, 485 grams in MZ1, and 479 grams in MZ2, demonstrating a lower weight at the end of the experiment of the exposed groups when compared to the control group (Figure 1).

Abdominal circumference was measured at the end of the experiment, showing evidence of statistical significance in the two groups exposed, MZ1 and MZ2, when compared to the control group, $p = 0.01$, with a median of 23 cm for the control group and 20 cm for the exposed groups.

Blood Count and Biochemical Parameters

Among the different blood count parameters, it was possible to detect a significant statistical difference in the platelet count of the exposed groups – (MZ1 $p = 0.003$), and (MZ2 $p = 0.015$) – compared to the control group (CG).

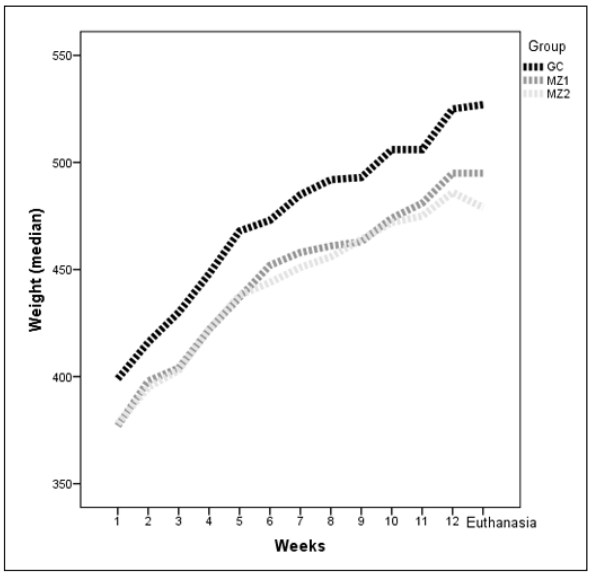

Figure 1. Median of the weight of animals in Groups GC, MZ1 and MZ2 over time.

Table I shows the different measurements of dispersion.

The FA enzyme revealed a significa ference in group MZ2 compared to the c ol group (p = 0.049), although no difference found in comparison to group MZ1.

There was a significant diffe tween th treated groups MZ1 and MZ2 gard he Ace- tylcholinesterase dosage, c ared to control group (p = 0.049). In e ting

aspartate aminotransferase (AST) and alanine aminotransferase (ALT), no significant difference could be detected (p = 0.23 and p = 0.90).

Biological Marker of Exposure – ET

ETU, as a biological marker of ex e, w evaluated and detected in the gr exposed with a median of 219 (ng/mL) in g MZ1, and of 587 (ng/mL) in group MZ2 show statis- tically significant exposure 0.05) ( ).

Genotoxicity

Genotoxicity was e ted ifferen sam- ples: liver tissue, l ma ood, a riph- eral blood.

Micronu

In the micronuc (MNs) count in bone marr d and per l blood (Figure 2), a st ally significant di ence was found $p \leq$ (when group MZ2 was compared to the CG (re 3). The n of (7.2 ± 1.1) micronuclei w bserved in oup MZ2, while in the con- trol (CG e mean was (1.0 ± 0.5). There was n nt statistical difference between roups MZ1 and MZ2.

Assay

The Comet Assay evaluation was performed in peripheral blood and liver tissue; there was a significant difference in the evaluation of the liver tissue of the groups exposed $p \leq 0.05$ (**Supplementary Figure 1**).

Table I. Comparativ of the blood and biochemical parameters.

	Control 9	MZ1 n = 8	MZ2 n = 9	*p*
Blood Co				
Hemog (mg/dL)	11.3-17.7)	17.7 (16.3-18.4)	17.4 (16.3-17.7)	0.150
Plate 10^5)	(4.85-11.21)[a]	11.60 (10.60-12.48)[b]	11.34 (9.87-12.72)[b]	**0.002**
W 10^3)	7.8 (4.7-9.1)	8.25 (6.3-10.1)	7.25 (5.7-8.9)	0.477
Ly yte)	7.0 (4.3-8.3)	7.3 (5.7-9.0)	6.3 (4.9-7.8)	0.421
Bioch				
T	69 (56-104)	69 (54-123)	56 (42-128)	0.238
	139 (121-204)	135 (112-309)	133 (90-291)	0.908
T bilirubin	0 (0-0.20)	0 (0-0.20)	0 (0-0.20)	0.989
D bilirrubin	0.08 (0.06-0.96)	0.05 (0-0.23)	0.05 (0.04-0.33)	**0.042**
in	-0.09 (-0.94 – -0.06)[a]	-0.05(-0.23-0)[b]	-0.05 (-0.31 – -0.04)[b]	**0.010**
cetylch sterase	433 (331-488)	480 (429-521)	497 (404-601)	**0.049**
Alkaline Phosphatase	150 (100-174)[a]	118 (98-169)[a,b]	116 (64-133)[b]	**0.049**
posure markers				
	-	219 (106-1041)	587 (232-1077)	0.059*

Data presented as median (minimum-maximum) and compared using the Kruskal-Wallis test. Different superscript letters represent astatistically diferente groups. *Mann-Whitney test. WBC: White blood cells AST: Aspartate Aminotransferase, ALT: Alanine Aminotrasnferase ETU: Ethylenethiourea.

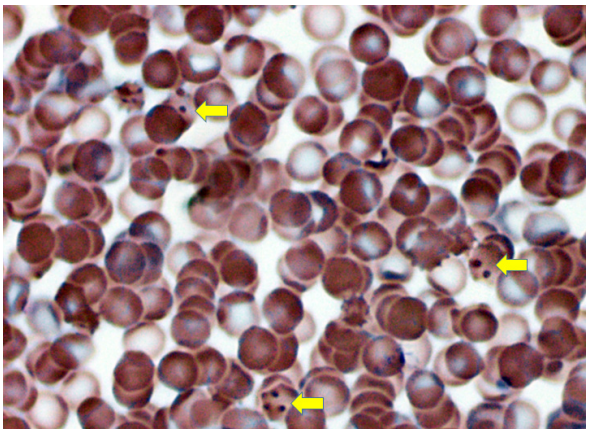

Figure 2. Optical microscopy photo HE 100×. Micronuclei in peripheral blood.

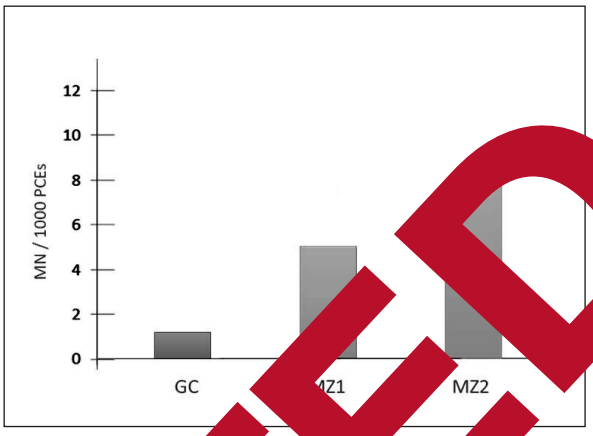

Figure 3. Micro us counts ne w blood, ***$p \leq 0.05$.

Oxidative Stress

When the statistical analyses of the oxidative stress markers were performed, we found a statistically significant difference in the Superoxide Dismutase (SOD), Catalase (CAT), and Reduced Glutathione (GSH) (Table II).

The results for thiobarbituric acid re e substances (TBARS) were not statistically icant when the groups were compared ($p >$).

In the CAT evaluation, there was statis significance when group MZ2 was compared the control group ($p = 0.011$).

In the analysis of SOD b en g ps MZ and MZ2, statistical si cance found (Mann Whitney $p = 0.02$

Reduced Glutathi (GS statistic y significant ($p = 0.0$ hen grou Z1 and MZ2 were compared CG. No sta l signifi-

canc ound in an e groups in evaluat-in bonylated proteins > 0.05).

tology

he histolo analysis of liver tissue, findings con ble with balloonization of the periver atocytes (A) and discrete perivenular inflammatory foci (B) (**Supplementary 2**), with a significant difference when the tion MZ1 and MZ2 groups were compared to the Control Group.

Histological patterns found: (**Supplementary Figure 3**)

A: Absence of portal fibrous, perivenular or perisinusoidal fibrosis.

B: Discrete Balloonization of perivenular hepatocytes, with occasional foci of inflammatory infiltrate.

Table xidativ ss.

	Control n = 9	MZ1 n = 8	MZ2 n = 9	*p*
dativ				
S (nm	0.47 (0.35-1.39)	0.45 (0.30-1.03)	0.58 (0.36-1.04)	0.388
St xide dis (SOD) (U D/mg prot)	25.4 (4.4-118.7)	35.8 (13.9-63.3)	15.5 (2.6-29.5)	0.086
moles/mi mg prot)	2.6 (2.2-2.9)[a]	2.4 (2.1-3.2)[a]	2.1 (1.7-2.2)[b]	**0.011**
Reduced glutatione (GSH) ols/min/mg prot)	0.085 (0.048-0.118)[a,b]	0.065 (0.041-0.073)[a]	0,088 (0,033-0.136)[b]	**0.020**
nylated proteins (n ol carb /mg pro)	2740.7 (1692.2-16996.8)	5370.0 (702.4-17119.7)	4697.1 (938.9-15701,1)	0.728

Date presented as median (minimum-maximum) and compared by the Kruskal-Wallis test. Different superscript letter represent satistically diferente groups.

C: Discrete Balloonization of the perivenular hepatocytes.
D: Discrete perivenular inflammatory foci.

Discussion

Exposure to different agricultural pesticides has become more frequent among the Brazilian population and worldwide. The purpose of this study was to analyze chronic exposure to Mancozeb and its toxic effect on health, mainly in the liver, using an experimental pilot model for a future clinical study.

It was demonstrated that 12-week exposure to Mancozeb led to a delay in weight gain throughout the experiment. There is little literature on measuring or approaching anthropometric measurements in a population exposed to agricultural pesticides. In this work, BMI was compared using the Lee Index, by means of multiple variables, and the result showed a statistically significant difference (p = 0.01) in the groups exposed, MZ1 and MZ2, compared to the control group. This result is supported by the difference in the abdominal circumference at the end of the experiment with animals in the groups exposed, which is always smaller after exposure to Mancozeb compared to the control group.

The evaluation of biochemical and hematological parameters, according Ar[illegible]d et al[1] (2017), showed results in which hepatological damage, expressed in [illegible] and [illegible] occurred; these modifications were not detected in this work. The authors[10] also describes alterations in the biochemistry of the liver, such as elevation of ALT, AST, alkaline phosphatase, and acetylcholinesterase activity, among the results observed in rats treated with Mancozeb, at 250 and 500 mg/kg for 4 weeks. Contrary to that study, the findings with statistical significance in the [illegible] were related to the platelet count in this study, showing an increased number of platelets in groups exposed (MZ1 and MZ2). Furthermore, there was a drop in the alkaline phosphatase levels in these groups, a finding in opposition with the results found in the literature, which are probably related to the nutritional component, clearly seen to be altered by exposure, suggesting malnutrition. Bowling presented studies[27,17] in which the alkaline phosphatase levels are low and suggested that they are related mainly to bone metabolism or some nutritional disorders.

Yahia et al[5], evaluating hepatic biochemical parameters, also found an elevation of the enzymes AST, ALT, alkaline phosphatase, and total bilirubin in a group of rats treated with 500 and 1,000 mg/Kg/day of Mancozeb, for 8 [illegible]. In this study, there was no statistically significant difference between the transaminases of the groups. Nevertheless, this fact does not invalidate the potential for damage.

The determination of ETU as a marker of exposure to the EBDCs has already been studied and proved by different authors[28,29] in clinical and experimental models. The experimental work enabled the determination of the effective evaluation by detection of ETU as a marker of exposure to Mancozeb. Aprea et al described ETU as a marker with a very rapid elimination kinetic, with maximum excretion within the first 24 hours. This experimental model was dosed several times beyond the 24 hours after the last exposure, and, even so, showed evidence of being a useful tool to evaluate exposure to EBDCs.

On the other hand, Fustinoni et al[29] present results of contamination in the control group, revealing levels in urine. The authors further confirmed the findings of this work, since they controlled the limitations of the external factors that occur in humans. The experimental model developed here enabled the detection of ETU levels in the urine of those exposed and did not show any evidence of ETU in the controls, validating the biological indicator of exposure in this sample.

In evaluating oxidative stress in liver tissue and serum, lipid peroxidation was analyzed by the TBARS technique; we did not observe a significant difference when the animals were exposed to the agent in groups MZ1 and MZ2, respectively. Other experimental models for cirrhosis and cancer, with xenobiotics such as utilizing DEN[30] and CCL4[31], observed increased lipoperoxidation by TBARS, different from our findings[30,31]. Other studies[24,32-35] have also shown evidence of greater lipoperoxidation in organs such as the kidney, lung and liver of animals that were cirrhotic through CCL4 or ligation of the bile duct. There was also an increase of lipoperoxidation in pictures of colitis through damage to the cellular membranes in an experimental model.

The antioxidant enzyme SOD is considered the first line of defense against the formation of EROS. The decrease of SOD activity in the MZ2 groups could be associated with the increase of TBARS that was consumed in an attempt to

diminish lipoperoxidation, and thus diminish oxidative damage based on the dismutation of the superoxide radical anions and formation of H_2O_2[35].

The significant increase of SOD enzyme activity ($p < 0.05$) in the animals of group MZ1 and MZ2 compared to the CG, suggests a protective effect after oxidative damage, which we can, in fact, observe from the lipoperoxidation (TBARS) damage, whose level is equal to those of the control group.

The function of CAT is to act on the H_2O_2, catalyzing it to water and O_2[36] In the present study, it can be observed that enzyme activity is diminished in the animals in groups MZ1 and MZ2. These data are in accordance with Schemitt et al[35], who observed that CAT was diminished in the livers of animals that presented liver damage induced by Thioacetamide.

Increased carbonylation of the liver proteins is associated with oxidative damage provoked by the aggressor agent, Mancozeb. Similar effects were observed with the use of Thioacetamide. In this scenario, the xenobiotic significantly increased the carbonyls, and increased carbonylation of the liver proteins is associated with oxidative [illegible] age[37]. On the other hand, using an antioxi[illegible] this case, melatonin, was linked to a signi[illegible]nt decrease. In this study, no evidence of a st[illegible] tically significant response was found (desp[illegible] different values among the gr[illegible]ch as [illegible] the findings of Atamaniuk et [illegible]n n[illegible]t can be suggested that the antioxi[illegible] enzym[illegible]acted as scavengers of the free rad[illegible] prot[illegible] lular membranes and [illegible]ven[illegible]peroxidat[illegible]n and the increased [illegible]onylation[illegible]ver proteins. The presence of [illegible]arbonyl gro[illegible]ldehydes, and ketones i[illegible] c[illegible]uence of t[illegible]oxidative damage ca[illegible]ed by agen[illegible]t attack the cellular membra[illegible]

Asi[illegible]om th[illegible] effects of [illegible]dative stress secondary to exp[illegible]e to Mancozeb, the evaluation of g[illegible]xi[illegible]can sho[illegible] chronic effects that are harmfu[illegible]humans[illegible]ese effects begin with [illegible]ar da[illegible]o[illegible]notoxic damage and potentially cause t[illegible]elopment of teratogenesis and ca[illegible][16,17], the g[illegible]notoxic potential being a primary[illegible]or long-term effects[10]. Genotoxicity [illegible]as evaluated using 2 different methods: Comet [illegible]ay and Micronucleus count. The methods were [illegible]ared and analyzed both in liver tissue and in peripheral blood and bone marrow blood. Among the results presented, a significant difference was observed in the analyses of liver tissue of groups MZ1 and MZ2, compared to the control group, and also in the bone marrow blood. It was possible to detect a statistical significance in group MZ2 compared to the control. According to the literature, the genotoxic potential is a risk factor for developing teratogenesis and cancer[16,17].

The histopathological findings in thi[illegible]K w[illegible] contribute to knowledge regarding [illegible] damage. It should be highlighted that th[illegible] very little literature that discusses th[illegible] histo[illegible]logical evaluation of the liver after [illegible]posure t[illegible]co-zeb. In this study, the fi[illegible]t its kind in [illegible] the evaluation was per[illegible]ed in [illegible]e rat liv[illegible]. The findings of this [illegible] w[illegible]balloon[illegible]zation and discrete peri[illegible]lar [illegible]matory [illegible]ltrate in the groups e[illegible]ed, with [illegible]evel[illegible]g into a severe lesion[illegible] was no fib[illegible] any of the samples, n[illegible]y [illegible]alterations [illegible]ggesting evolution to advanced l[illegible] disease, probably due to the ti[illegible]exposure. [illegible] literature, the study by[illegible]es et al[9] showed ev[illegible]nce of an increased i[illegible]ence of adenoma and hepatocarcinoma in r[illegible]treated for [illegible]onths, with a time of exposure[illegible] times gre[illegible] than in this study.

[illegible]nform[illegible]n is useful, however, in the presen[illegible]e main reason why the time of 12 weeks was chosen was to mimic real life in a pilot [illegible] considering that the workers are exposed t[illegible]oduct (Mancozeb) for approximately 2 to 3 months, during the cultivation period, after which they stop and only resume their activities a long time later. In no case is the exposure continuous for longer than 6 months. This was based on the duration of the life of a rat under animal research laboratory conditions. These animals live an average of 18 months (547.5 days), and when this period is converted into years of life, 12 weeks (84 days) correspond to approximately 11 years of life, a reasonable time length when considering chronic exposure[38].

Limitations

The main limitation of the study was having to perform the exposure to Mancozeb by gavage, and not by inhalation since gavage is the only method approved by the Research Ethics Committee. As described in the objectives of this work, the idea was to mimic real life, however, following the guidelines and normativity in force in the animal experimentation unit, the use of exhaustion hoods for exposure by inhalation, in order to protect the research team was not approved.

This study was carried out during the COVID-19 pandemic, a circumstance that conditioned its development and also became a lim-

itation, due to the reduction in the operating hours of the animal experimentation unit and restrictions of the researchers, who were allowed access only in small groups or even individually, to respect the recommendations and protocols of the hospital infection control center in order to avoid the proliferation of the virus.

A further limitation was the small sample size, defined in accordance with the current legislation in Brazil (Law 11,794 of October 8, 2008), which establishes procedures for the scientific use of animals and follows regulations of the humane use of animals from the normative resolutions n° 30/2016 (Brazilian Guideline for the Care and Use of Animals in Teaching or Scientific Research Activities - DBCA), and n° 37/2018 (Guidelines of Euthanasia Practice) of the National Council for the Control of Animal Experimentation – CONCEA.

Conclusions

The results confirmed the efficacy of the experimental model to induce hepatotoxicity. In the animals treated, Mancozeb could alter aspects ranging [illegible] anthropometric measurements to liver histolo[illegible]

After developing an experimental model [illegible]-icking the reality encountered in the countr[illegible] terms of agriculture and grain production, w[illegible] the consequent use of chemic[illegible] as pest[illegible] cides, specifically Mancozeb, wa[illegible] ncluded that this pesticide is prej[illegible]al to h[illegible]n, espe-cially to DNA; this was [illegible]nstrat[illegible] of blood tissue from [illegible]bo[illegible]w and liver tissue of the rats st[illegible]d.

The study d[illegible]d is a pi[illegible]odel, the beginning of [illegible]rge[illegible] of researc[illegible]elated to chronic ex[illegible]sure to agr[illegible]ural pesticides, continuing t[illegible]inical model [illegible]eeks to evaluate the ef[illegible]of Mancozeb on vit[illegible]ture.

Conflict of Interest

[illegible]of the [illegible]s ha[illegible]mpeted for financial interests or pe[illegible]l relati[illegible]t could have appeared to influence the [illegible]k reporte[illegible]his paper.

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Authors' Contribution

N.D Suarez Uribe, M.F Pezzini and D. Joveleviths designed and coordinated the study; J. Dall Agnol, N. Marroni, S. Benitez, D. Benedetti, J. da Silva, C.T. Cerski, E. Dallegrave, S. Macedo performed the experiments, acquired and analyzed data; N.D Suarez Uribe, M.F Pezzini and D. Joveleviths interpreted the data; N.D Suarez Uribe, M.F Pezzini wrote the manuscript; all authors approved the final version of the article.

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Data Availability

T[illegible]tasets genera[illegible]during and/or analyzed during the cu[illegible]study are a[illegible]ble from the corresponding author upo[illegible]est.

Approval

This [illegible]y was submitted to and approved by the Medical Ethics Committee of Hospital de Clínicas de Porto Alegre, HCPA, under number 2019-0647.

Informed Consent

Not applicable.

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